Circular Dichroism Spectropolarimeter Instructions

Circular Dichroism

Circular dichroism (CD) spectroscopy measures the difference in the absorption of left-handed and right-handed circularly polarized light. In general, the CD phenomenon will occur for any optically active molecule. Importantly the CD signal for proteins and DNA is imparted by the secondary structure, which has chirality (right-handed versus left-handed helix for example). Thus CD is a good technique to measure the secondary structure in proteins.

CD spectroscopy is good for:

- Determining if a protein is folded
- Determining the percent helix, sheet, turns, and random structure in proteins
- Comparing the secondary structure of proteins in different conditions (temp, pH, salt, concentration, ligands, etc.)
- Measuring the protein stability through thermal melts and denaturation studies
- Testing the structural integrity of site directed mutations
- Testing the stability of domain structures

Scheduling

- Schedule CD from the Faces Scheduling System (http://faces.ccrc.uga.edu). Use UCHC_NMR as the Group and your username and password.
    - NOTE: Contact Mark Maciejewski (markm@neuron.uchc.edu) if you need an account

Starting the instrument

1. **Turn on N_2 gas and adjust regulator to provide 7 lpm flow rate.**
    a. NOTE: At 7 lpm approximately 1,000 kPa are depleted in one hour.
    b. NOTE: Nitrogen is cheap, $5.00 per tank, don't push the tank to the end
    c. NOTE: You can switch N_2 tanks while running the CD but act quickly. Shutdown the instrument if something goes wrong and you cannot restore the N_2 purge gas within a few minutes.
    d. NOTE: Running the CD for any significant length of time (longer than 5 minutes) without a nitrogen purge will damage the optics and will result in up to a $5,000 repair.
2. Turn on the cooling water bath
    a. Do not change the set temperature
    b. NOTE: This does not control the sample temperature
3. **Wait 20 minutes to completely purge the system with N_2 gas**
4. Turn on the orange power switch and wait 10 seconds
5. Turn on the green power switch
6. Log the starting lamp hours in the logbook. The timer is located on the power supply.

7. Turn on the temperature controller and hit the START button.
    a. Do not touch other buttons.
8. Start the "**Spectra Manager**" software
9. Set the sample temperature (see the next section for details)
10. Allow 20 minutes for instrument to properly warm-up.
11. Measure the photomultiplier voltage at 300 nm with an empty sample chamber (make sure the spacer is removed) and a 1 nm bandwidth and log the voltage in the logbook.

Setting sample temperature

1. Go to Measurement → Accessory → Temperature and select Jasco Peltier
2. Go to Control → Accessory → Enter desired temperature → Apply

Quick Instrument settings

Detailed description of instrument settings are below, but for quick setup these settings should work for most samples.

<u>Wavelength Scan</u>

- 0.1 mm pathlength
- 0.2 mg/ml protein concentration
- 225 µl volume
- Dilute buffers and salts
- 1.0 nm bandwidth
- 0.5 nm resolution
- 16 scans (more if signal is weak)
- 4 sec response
- 20 nm / min scan speed
- Scan range 280 nm to as low as possible while keeping HT voltage less than 700 V

<u>Thermal Melt</u>

- 1.0 cm pathlength (can use mini stir bar)
- 0.02 mg / ml protein concentration
- 2.0 ml volume
- Very dilute buffers and salts
- 2.0 or 5.0 nm bandwidth
- 222 nm wavelength
- Initial temperature 4 deg C
- 16 sec response
- Temperature slope of 35 deg C per hour from 4 to 90 deg C
- Repeat data collection from 90 to 4 deg C.

Shutting down the instrument

1. Exit the "**measurement"** program that you were running
2. In "**Spectra Manager"** shutdown the instrument by going to Instrument → Stop and then close the "**Spectra Manager"**

3. Turn off the temperature controller
4. Turn off the water bath
5. Turn off the green power switch on the power supply
6. Turn off the orange power switch on the power supply
7. Record the ending lamp hours in the logbook
8. Turn off the N_2 gas

Detailed instrument settings

Band Width

- A bandwidth of 1 nm is typical. However using 2 nm can be beneficial for weak samples. Larger bandwidth allows more light helping sensitivity, but hurting resolution.

Step resolution

- Typical step resolutions are 0.2 nm to 1.0 nm. Step resolutions 50% of the bandwidth are normal.

Number of accumulations

- The S/N is related to the number of accumulations and the greater the number of accumulations the higher the signal-to-noise, but at the cost of a longer experiment time.
- Using the chart below the number of accumulations needed for various CD signal sensitivities can be estimated
    - NOTE: To test the CD signal place sample in instrument and set wavelength to 222 nm.
    - NOTE: If different response and scan rates are used the number of accumulations may need to be adjusted.

$$\frac{Signal}{Noise} \propto \sqrt{Number\ of\ accumulations}$$

Recommended number of accumulations at a 2 sec Response and 20 nm/min scan rate based on CD sensitivity at 222 nm

CD Sensitivity (mdeg)	Number of Accumulations	Relative Signal-to-Noise
100	**2**	**141**
50	**4**	**100**
20	**8**	**57**
10	**16**	**40**
5	**32**	**28**
2	**64**	**16**
1	**128**	**11**

Response

- The accumulation time per data point.
    - Having a larger response will give better S/N but if you are performing a wavelength scan, the scan rate and response must be set appropriately. See table below.

$$\frac{Signal}{Noise} \propto \sqrt{Response}$$

        - Thus a response of 4 seconds will have twice the S/N as a response of 1 second
    - For thermal melts the response time can be set high as the wavelength is not changing and the temperature should be increasing slowly.

Scanning speed

- Scanning speed is how fast the wavelength scan occurs and should be adjusted in consideration with the response.
- Typical scan speeds for proteins are 20 to 100 nm/min

Recommended minimum and maximum Response times for various scan speeds

Scanning speed (nm/min)	Minimum Response	Maximum Response
2	4.0 sec	16.0 sec
5	2.0 sec	8.0 sec
10	2.0 sec	4.0 sec
20	**1.0 sec**	**4.0 sec**
50	**0.5 sec**	**2.0 sec**
100	**0.25 sec**	**1.0 sec**
200	0.125 sec	0.5 sec
500	64 msec	0.25 sec
1000	32 msec	0.125 sec

Saving data

Sample preparation

Buffers

- Most buffers are not optically active and therefore do not give rise to a CD signal. However, many buffers and salts absorb strongly in the far-UV. When collecting CD spectra it is advantageous to go to as low a wavelength as possible (< 200 nm), but that can only be achieved if the total absorption in the far-UV is low (see HT / PM voltage section below). This can be achieved by using low concentrations of salts and buffers, using smaller path length cells, and choosing buffers and salts that do not absorb as strongly.

- Good buffers include phosphate (pH 6-8), and borate (pH 8-10). Buffer concentrations should be kept low (typically 1 to 5 mM). High quality Tris (Trizma) is okay. Other organic buffers should be used only when absolutely necessary.

Salts

- Chloride ions absorb strongly in the far-UV so keeping NaCl and KCl concentrations low is beneficial. NaF is a good alternative to NaCl. To minimize chloride ions it is a good idea to make phosphate buffers by mixing di- and mono-sodium phosphate together to achieve the proper pH rather than adjusting the pH with HCl.

Other buffer components

- Imidizole, DTT, DMSO, glycerol, detergents, and other items should be removed when preparing samples to reduce the total absorbance in the far-UV.

Protein concentration and volumes

- The CD signal arises from the peptide bonds and therefore larger proteins will give a stronger CD signal than a smaller protein at the same concentrations. This is why the size of the protein is used when determining molar ellipticity values. It is also the reason that CD concentration recommendations are in mg/ml rather than μM.
- For CD scans with a 0.1 mm path-length protein concentrations should be around 0.1 to 0.3 mg/ml and a total volume around 225 μl
- For thermal melts with a 1.0 mm path-length protein concentrations should be around 0.01 to 0.03 mg/ml and a total volume of 2.0 ml

HT (photo-multiplier) voltage

- The CD instrument has a HT voltage reading that is shown on the screen and recorded with the data. This HT voltage is a measure of how much voltage the detector is using and is dependent on the amount of light striking the detector (more light – lower HT voltage). At lower wavelengths the protein, buffer, water, and air will all absorb light and less will strike the detector causing the HT voltage to rise. Once the HT voltage goes above 700 V there is too little light striking the detector to make an accurate measurement and the data at those wavelengths should be discarded.
- Before performing a wavelength scan the wavelength should be adjusted manually to find the lowest wavelength where the HT voltage is below 700 V and then that wavelength used as the lowest wavelength in the scan.

Cleaning cuvettes

1. Remove sample
    a. Using suction is a convenient way to remove samples
2. Rinse with H_2O
3. Fill with nitric acid (HNO_3) or HCl for 5-10 minutes and remove

    a. Be careful, as HNO_3 is corrosive and dangerous
4. Rinse thoroughly with milli-Q H_2O (10X)
5. Final rinse with HPLC grade methanol
6. Allow all methanol to evaporate
    a. Never used compressed air from the wall to dry cuvettes, as small amounts of oil are likely to get onto the glass.
    b. Compressed air in cans is fine.
7. Overnight soaks in HNO_3 or HCl are useful on occasion to thoroughly remove protein bound to the glass.

Converting mdeg to Molar Ellipticity

$$[\theta](deg \cdot cm^2 \cdot dmol^{-1}) = \frac{Ellipticity\ (mdeg) \cdot 10^6}{Pathlength\ (mm) \cdot [Protein](\mu M) \cdot (n-1)}$$

where n is the number of residues in the protein.

NOTE: The protein concentration is critical when determining accurate molar ellipticity values. Use the Edelhoch method for accurate protein concentrations using UV screctoscopy.

Edelhoch Method of Protein Concentration

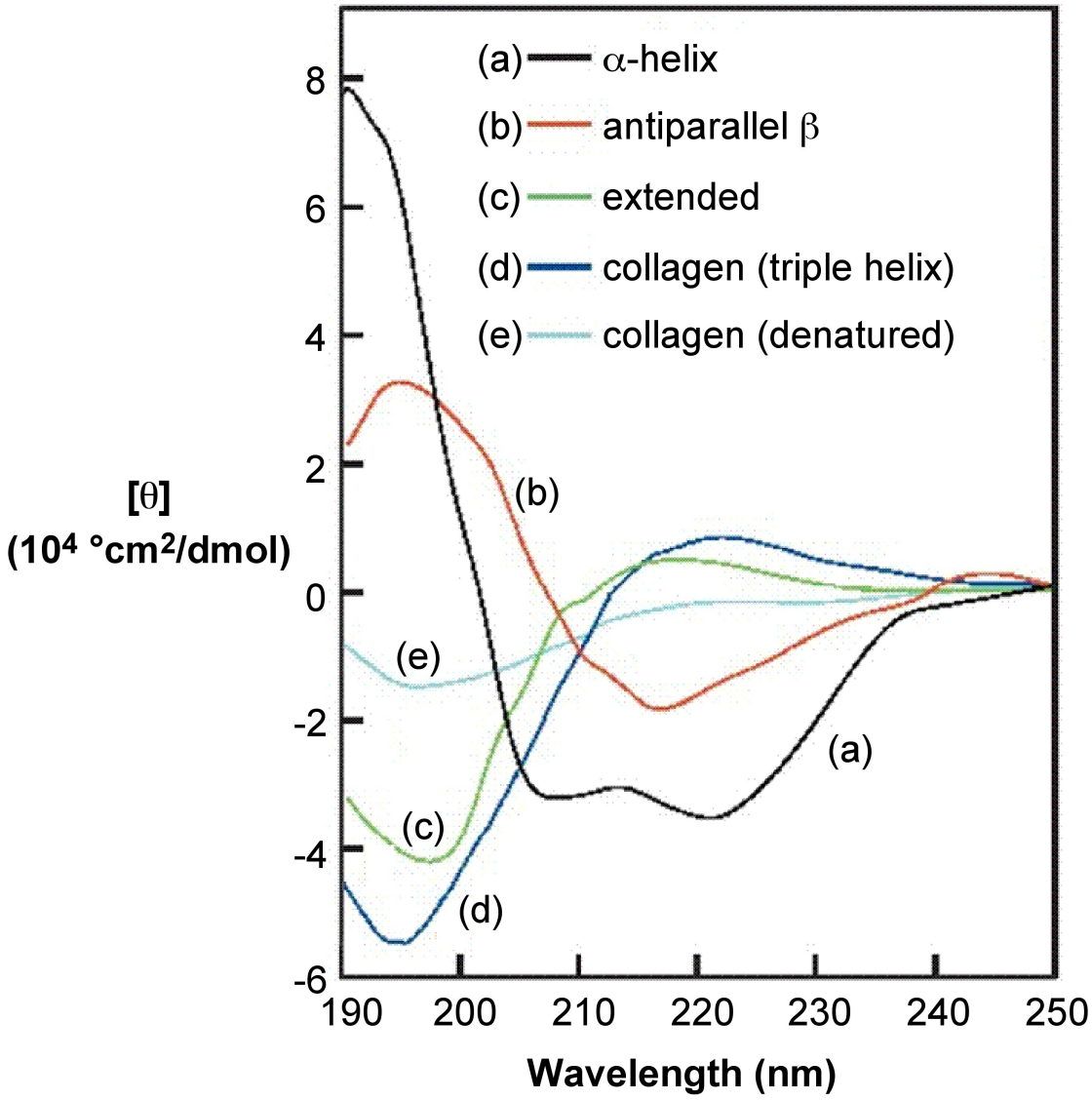
- Count the number of Trp, Tyr, and Phe residues in your protein and determine the molar extinction coefficient at 280 nm in $M^{-1}\ cm^{-1}$ using:

$$\varepsilon_{280} = (5500 \times n_{TRP}) + (1490 \times n_{TYR}) + (125 \times n_{S\text{-}S})$$

  where n_{TRP}, n_{TYR}, and $n_{S\text{-}S}$ are the number of TRP residues, TYR residues, and disulfide bonds in the protein, respectively.

- Measure the absorption at 280 nm of the protein solution in 20 mM phosphate buffer, pH 6.5 with 6 M guanidine-HCl. Ensuring that the total absorption at 280 nm is between 0.1 and 1.0 where the absorption is linear.

Typical CD spectra of alpha helix, beta sheet, and random coil



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